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Biochimica et Biophysica Acta 1715 (2005) 37 – 48
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
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Preparation and in vivo evaluation of PEGylated spherulite formulations

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Received 4 March 2005; received in revised form 23 June 2005; accepted 28 June 2005
Available online 25 July 2005

Abstract

Spherulites are multilamellar vesicles obtained by shearing a lamellar phase of lipids and surfactants. They consist of concentric bilayers of amphiphiles alternating with layers of aqueous medium in which hydrophilic drugs can be sequestered with high yield. To be useful for drug targeting applications, spherulites should be small and long circulating. The objectives of this work were threefold. First, the spherulite size was optimized to obtain a mean diameter of less than 300 nm. Second, the vesicle composition was adjusted to minimize in vitro leakage of internal content. Third, the spherulites were coated with 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-[methoxy poly(ethylene glycol)] (DSPE-PEG) to impart them with a long half-life. Then, the PEGylated spherulites (Phospholipon 90G/Solutol HS15/cholesterol/DSPE-PEG 2000 or 5000) were loaded with 1-β-D-arabinofuranosylcytosine (ara-C) and injected intravenously to rats. They were compared to uncoated spherulites and to an ara-C solution. The surface-modified vesicles exhibited long circulation times with areas under the blood concentration vs. time curve exceeding by 3.1- to 6.9-fold that of uncoated spherulites. Similarly, blood levels of ara-C encapsulated in PEGylated vesicles were higher than those of the controls, but they did not parallel the carrier pharmacokinetics. Two hours post-injection, most of the drug was cleared from the systemic circulation, reflecting rapid leakage of ara-C from the vesicles.

Keywords: Spherulite; Liposome; Ara-C; Biodistribution

1. Introduction

Colloidal carriers have been investigated mainly for the delivery of anticancer agents to solid tumors after intravenous (IV) administration. Liposomes represent the most studied particulate drug carriers and are now considered to be a mainstream drug delivery technology. However, without appropriate surface modifications, these vehicles may not be suitable for passive accumulation of cytostatics into tumors, partly due to adsorption of plasma proteins (opsonins) onto the phospholipid membrane, triggering recognition and uptake of the liposomes by the mononuclear phagocytic system (MPS) [1]. Besides, a variety of blood proteins are known to adsorb onto the carrier in vitro and in vivo [2–4], some of which can cause membrane destabilization and early leakage of entrapped content [5–7].

A major advance in liposome technology came with the advent of Stealth® carriers, a technology which relies on surface coating with a flexible, hydrophilic polymer, usually a lipid derivative of poly(ethylene glycol) (PEG), to provide steric stabilization of the vesicle. The highly hydrated PEG corona on the liposome surface can reduce interactions between cells and lipid head groups, act as a barrier to the binding of opsonins [3,8,9] and/or hinder the association of liposome-bound opsonins with macrophage receptors [10]. In return, the zone of steric hindrance created by the hydrophilicity and the chain flexibility of PEG slows down liposomal clearance by the MPS, and consequently increases their localization in solid tumors [11,12]. The restricted binding of serum opsonins promoted by PEG chains, also prevents early leakage of the encapsulated molecule [5,6].

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0005-2736/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2005.06.013

Liposomes rely on passive targeting to increase the localization of anticancer drugs in the vicinity of tumoral cells. Growing tumors possess vasculature with enhanced permeability as a result of the disease process [13,14]. Pore diameters in tumor capillaries can range from approximately 100 to 400 nm [15,16]. Therefore, drug-loaded liposomes must be small enough to extravasate from the blood into tumor interstitial space through these pores [17]. Furthermore, particle size was shown to be critical in achieving long circulation times; an inverse correlation exists between liposome size and uptake by macrophages [18] or circulation time in vivo [15].

One of the major drawbacks of liposomes remains their relatively low entrapment efficiency (EE) [19]. Successful approaches have been developed to increase the encapsulation yield of many antineoplastic drugs. Indeed, amphiphilic drugs that are weak bases or weak acids can be loaded into the liposome core using remote loading procedures like the ammonium sulphate and pH-gradient methods for doxorubicin [20] and vincristine [21], respectively. These approaches are generally associated with much higher EE. However, for drugs that are poorly or not at all ionizable or that exhibit a high molecular weight, remote loading methods may not be appropriate. Furthermore, most liposomal preparation methods require the use of organic solvents, which need to be removed from the formulation during the process [22].

Recently, a new solvent-free process to prepare well defined multilamellar vesicles (MLV) with high encapsulation efficiencies has been described [23,24]. Diat et al. [23,24] discovered that moderate shearing of a lyotropic lamellar phase of surfactants in the presence of a minimal amount of water could lead to the formation of MLV (Fig. 1). Moreover, the overall polyhedral structure of these multilayered vesicles was conserved upon dilution [25]. The MLV so formed have been referred to as Spherulites®. The particle size can be controlled precisely by varying the shear rate [23,24] and the components in the preparation [26]. Like

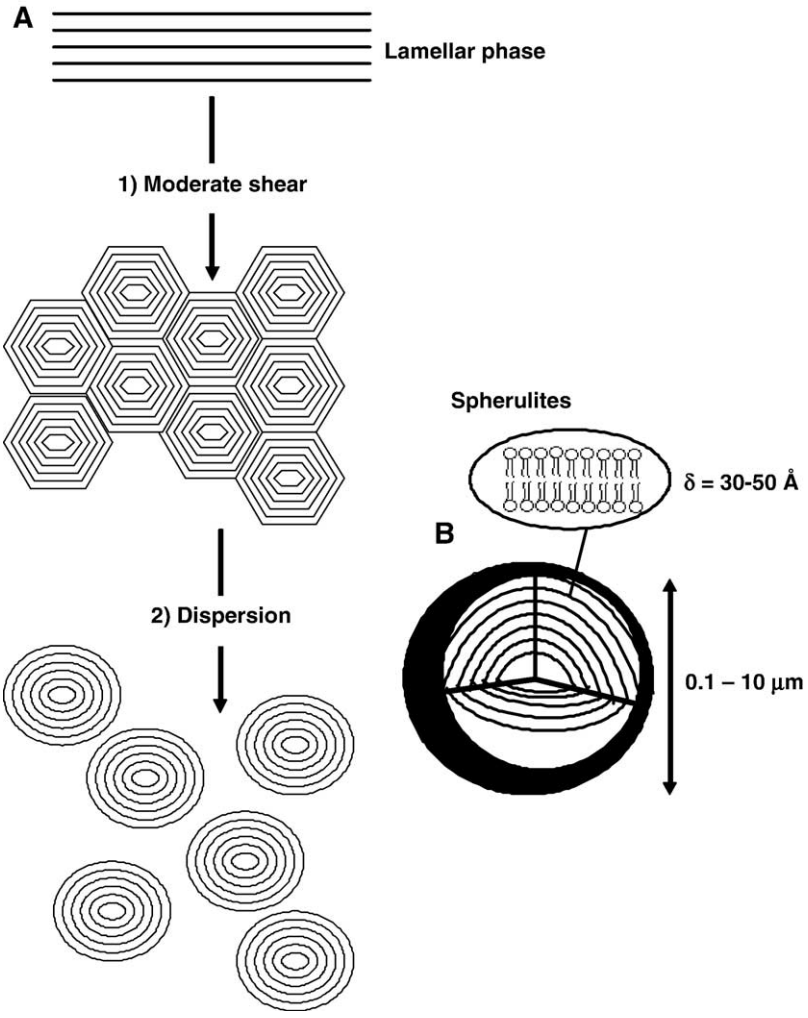

Fig. 1. Process of preparation of spherulites in suspension (A) and a schematic representation of the spherulites structure (B). Spherulites are produced by applying a moderate shear to a lamellar phase of surfactant. The lamellar phase forms a close-packed organization of MLV in which regular stacking of surfactant bilayers ($n = 50 – 1000$ layers) are separated by aqueous layers. Compacted spherulites can be dispersed by adding an excess amount of solvent. The interlamellar distance between two constitutive layers and the bilayer thickness are typically between 50 and 200 Å. Reproduced from ref. [31] with permission.

liposomes, spherulites have rapidly found many applications as encapsulating systems [26–29]. Both are composed of phospholipids, except that the structure of spherulites is made of uniformly spaced concentric bilayers of amphiphiles alternating with layers of aqueous medium. The interlamellar distances between two constitutive layers and the bilayer thickness are always constant within a single vesicle as evidenced by X-ray diffraction analysis. However, the interlamellar distance may slightly increase upon the encapsulation of compounds such as short DNA fragments [30] and copper (II) ions [31]. This organized structure remains very stable in the dispersion medium [26,28,31] and confers high EE for a variety of compounds, such as copper salts [31], fluorescent dye [29], and macromolecules like proteins [27] and DNA [26,32].

Spherulites, as described in the literature, have diameters typically around 1 μm [25,31] and are not suitable for drug targeting applications. Moreover, no pharmacokinetic study involving PEGylated spherulites has been published so far [33]. To be useful as drug delivery agents, these vesicles should exhibit a small size (<300 nm) and have a long circulating time. Accordingly, the objectives of this work were threefold. First, spherulites were prepared using amphiphiles approved for parenteral administration, and their size was optimized to obtain a diameter of less than 300 nm. Second, the vesicle composition was adjusted to minimize in vitro leakage of a model fluorescent dye. Third, the spherulites were coated with 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-monomethoxy-[PEG] (DSPE-PEG) to confer long circulation times to the vesicles after IV injection. In this study, we examined the effect of PEG chain length on the spherulite pharmacokinetic parameters and on the transport of a hydrophilic anticancer agent, i.e. 1-β-D-arabinofuranosylcytosine (ara-C). This drug, commonly used in the treatment of acute myelogenous leukaemias [34] represents an interesting model for delivery by sustained release systems due to its short biological half-life (16–20 min) [35,36].

2. Material and methods

2.1. Materials

Phospholipon® 90 G (P90) (94% of soybean phosphatidylcholine, PC) was a gift from Rhône-Poulenc Rörer (Köln, Germany). Lipoid® S75 (70% of soybean PC) was kindly provided by Lipoid GmBH (Ludwigshafen, Germany). Solutol® HS15 (PEG 660 12-hydroxystearate) was a gift from BASF (Ludwigshafen, Germany). DSPE-PEG 2000 and cholesterol (Chol, 99.5% pure) were obtained from Northern Lipids Inc. (Vancouver, BC, Canada). L-α-dioleoy-phosphatidylethanolamine (DOPE) and DSPE-PEG 5000 were from Avanti Polar Lipids (Alabaster, AL). Tween® 20 (polyoxyethylene 20 sorbitan monolaurate), Tween® 80 (polyoxyethylene 80 sorbitan monooleate), 2,4,6-trinitrobenzenesulfonic acid solution (TNBS) (1.7×10^{-1} M), Triton X-100, Sepharose® 2B, and ara-C were purchased from Sigma (St. Louis, MO). Trisodium 8-hydroxypyrene trisulfonate (HPTS) and *p*-xylene-bis-pyrimidium (DPX) were obtained from Molecular Probes (Eugene, OR). Sephadex® G-50 and G-100 were from Pharmacia Biotech (Uppsala, Sweden). [^{14}C]-Cholesteryl oleate (52 mCi/mmol) and [^{3}H] -ara-C (33 Ci/mmol) were purchased from Perkin Elmer (Boston, MA) and Amersham Pharmacia Biotech (Uppsala, Sweden), respectively. All products were used without further purification. Water was deionized with a MilliQ purification system (Millipore, Bedford, MA) before use.

2.2. Preparation of spherulites

Spherulites were prepared according to the procedure of Mignet et al. [26]. The lecithins and hydrophilic surfactants (Solutol HS15 or Tween) were precisely weighed and mixed. Then, a solution of NaCl 0.9% (w/v) was added and the mixture was hydrated overnight at room temperature. Spherulites were obtained through manual shearing of the lyotropic lamellar phase. Polarized light optical microscopy (Axiovert S100, Carl Zeiss Canada ltée, Kirkland, QC, Canada) was used to confirm the structural properties of the vesicles. The lamellar phase presented a homogeneous birefringent texture characterized by Maltese crosses (Fig. 2A) [23,24]. Dilution of this preparation in saline led to the dispersion of vesicles without changing their structure, as ascertained by the presence of Maltese crosses. Formulations containing Chol were prepared using a similar method except that the lipid components were first dissolved in chloroform. The solvent was evaporated under reduced pressure, and the resulting film was further dried in vacuo (~0.1 mBar) for 30 min. The composition of all prepared formulations is listed in Table 1.

2.3. Incorporation of DSPE-PEG

PEGylated spherulites were prepared by incubating preformed vesicles with DSPE-PEG (15 mg/mL) micelles for 1 h at 40 °C, as described elsewhere for conventional liposomes [37]. This procedure allows PEG insertion in the outer leaflet only. The concentration of PEG represented 10 mol% of phospholipids exposed on the outside surface of spherulites (less than 1 mol% of total phospholipids). The amount of surface-exposed phospholipids was determined by the procedure of Barenholz et al. [38]. Spherulites (c.a. 280 nm) with 1% (w/w) DOPE were prepared and diluted with saline to a final volume of 0.6 mL (3.1 mg/mL total lipids/surfactant). Then, $NaHCO_3$ solution (0.2 mL, 0.8 M, pH 8.5) was mixed with the vesicle suspension. Twenty μL of 1.5% (w/v) TNBS were added and allowed to incubate in the dark for 30 min at ambient temperature. After the incubation period, 0.4 mL

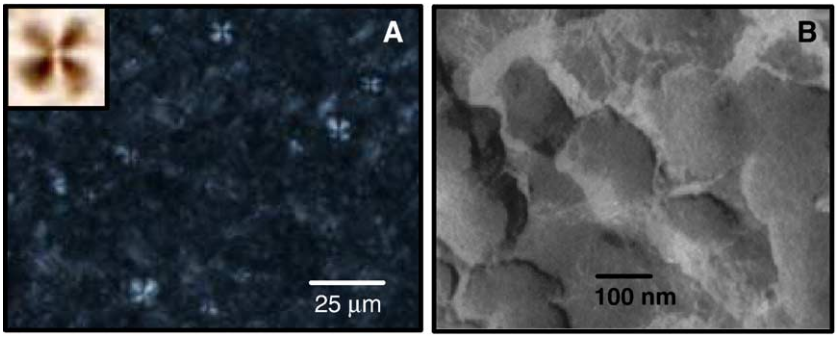

Fig. 2. Polarization (A) and freeze-fracture electron micrographs (B) of a manually sheared spherulite sample (formulation 11). These non-diluted vesicles had a typical size about 200 nm and presented the birefringent texture.

of 2% (v/v) Triton X-100 in 1.5 N HCl was added to the sample to stop the reaction. The absorbance (λ=410 nm) was measured on an Ultrospec 2000 spectrophotometer (Amersham Pharmacia Biotech, Uppsala, Sweden) within an hour after acidification. The total DOPE content was determined by the same procedure except that spherulites were first solubilized with 2.6% (v/v) of Triton X-100.

2.4. Freeze-fracture electron microscopy

The sheared lamellar phase was quenched using sandwich technique and liquid nitrogen-cooled propane. Using this technique, a cooling rate of 10,000 °K/s is reached avoiding ice crystal formation and artefacts possibly caused by the cryofixation process. The cryo-fixed samples were stored in liquid nitrogen for less than 2 h before processing. The fracturing process was carried out in JEOL JED-9000 freeze-etching equipment and the exposed fracture planes were shadowed with Pt for 30 s in an angle of 25–35° and with carbon for 35 s (2 kV/60–70 mA, 0.13 nBar). The samples were cleaned with concentrated, fuming HNO_3 for 24 h followed by repeating agitation with fresh chloroform at least 5 times. The replicas cleaned this way were examined with a JEOL 100 CX electron microscope (Jeol USA, Peabody, MA).

2.5. Particle size analysis

Particle size was determined at ambient temperature by dynamic light scattering (DLS) at a 90° angle on a Coulter N4Plus (Coulter Electronics, Miami, FL) employing differential size distribution processor analysis. Measurements of the mean hydrodynamic diameter were performed in triplicate.

2.6. In vitro release of encapsulated compounds

2.6.1. Fluorescent dye (HPTS)

Spherulites were prepared as mentioned above except that the saline solution was replaced with a buffered solution of *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) (20 mM, pH 7.4) containing the water-soluble fluorophore HPTS (35 mM) and the collisional quencher DPX (50 mM) [39]. Untrapped dye was removed by gel filtration over a Sephadex G-100 column (1 × 30 cm). The release of spherulite content was monitored at 37 °C in HEPES buffer for 270 min at 37 °C by a fluorescence dequenching assay on a Series 2 Aminco Bowman fluorimeter (Spectronics Instruments Inc, Rochester, NY). The extent of content release was calculated from HPTS fluorescence intensity (λ_{ex}=413, λ_{em}=512 nm) relative to measurement after vesicle disruption in 0.9% (v/v) Triton

Table 1
Spherulite formulations and mean hydrodynamic diameters obtained by DLS

Formulation	P90 (% w/w)	Lipoid S-75 (% w/w)	Solutol HS15 (% w/w)	Tween 20 (% w/w)	Tween 80 (% w/w)	Chol (% w/w)	NaCl(aq) (% w/w)	Mean Size±S.D. (nm)
1		23			42		35	no spherulite
2		19	36				45	no spherulite
3		42			23		35	219±38
4	42				23		35	190±13
5	45		20				35	336±42
6	42		23				35	238±23
7	36		29				35	232±31
8	34		31				35	183±37
9	38		25				37	247± 65
10	42			13		10	35	216±37
11	42		13			10	35	298±26
12	34		11			20	35	no spherulite

X-100, which gave complete release of encapsulated HPTS and DPX.

2.6.2. Ara-C

Lipids spiked with [^{14}C]-cholesteryl oleate (57 pCi/mg total lipids/surfactant) were hydrated with a saline solution of ara-C (2% w/w of total lipids/surfactant) spiked with [^{3}H]-ara-C (114 pCi/mg total lipids/surfactant) and the spherulites were prepared as described above. Drug loading was determined by radioactivity counting after separation of free ara-C from encapsulated ara-C by gel filtration over a Sephadex® G-50 (1.5 × 20 cm) column. Radioactivity was measured in a scintillation counter (Liquid Scintillation Analyser Tri-Carb 2100TR, Packard, Meriden, CT) after the addition of Hionic Fluor® scintillation cocktail. The EE was calculated using Eq. (1):

$$\mathrm{EE}(\%) = \frac{\mathrm{AUC_E}}{\mathrm{AUC_T}} \times 100 \quad (1)$$

where $\mathrm{AUC_E}$ and $\mathrm{AUC_T}$ stand for area under the elution profile curve of the encapsulated and total drug in the feed, respectively.

The release of ara-C was assessed after incubation of radiolabelled spherulites in 50% (v/v) fresh rat serum at 37 °C for 15 min. Released ara-C was separated from spherulites and excess serum components by gel filtration over a Sepharose® 2B column (1 × 30 cm) and assayed by radioactivity counting. The percentage of ara-C released was calculated with Eq. (2).

$$\text{Ara-C released } (\%) = \frac{\mathrm{AUC_F}}{\mathrm{AUC_T}} \times 100 \quad (2)$$

where $\mathrm{AUC_F}$ represents the area under the elution profile curve of the released drug.

All experiments were conducted in triplicate.

2.7. In vivo pharmacokinetics and biodistribution

In vivo studies were carried out using male Sprague–Dawley rats (300–350 g) (Charles River, St-Constant, QC, Canada). The studies were approved by the Animal Welfare and Ethics Committee of the University of Montreal. The rats were surgically prepared for IV administration and arterial blood sampling, as previously described [40]. Briefly, polyethylene catheters were inserted into the femoral vein and artery, protected with a tethering system, and the rats were allowed to recover for at least 24 h. Ara-C-loaded spherulites labelled with [^{14}C]-cholesteryl oleate and [^{3}H]-ara-C were prepared as described in Section 2.6.2.

The rats were subdivided into 4 groups (5 rats/group). The first group received only a saline solution of ara-C spiked with [^{3}H]-ara-C, whereas the second, third and fourth groups were injected with ara-C loaded-spherulites that were respectively non-PEGylated or coated with 10 mol% of DSPE-PEG 2000 or 5000 (P90/Solutol HS15/Chol/DSPE-PEG, 57.4:14.8:27.2:0.6 mol%). The formulations (400 μL) were injected via the vein cannula with 0.33 μmol/kg of lipids, corresponding to 2.34 μg/kg ara-C, 6.2 μCi/kg of [^{3}H]-ara-C and 5.7 μCi/kg of [^{14}C]-cholesteryl oleate. Blood samples (400 μL) were collected at 5, 15, and 30 min, and 1, 2, 4, 8, 12, and 24 h post-injection. The rats were sacrificed after the last blood sampling point, weighed and perfused with saline, prior to harvesting liver, lungs, kidneys, spleen, and heart. Blood and tissues were weighed and treated with Soluene 350® (Camberra Packard, Mississauga, ON, Canada). After digestion, blood samples were bleached by successive additions of hydrogen peroxide (H_2O_2, 30% v/v). The samples were left to stand in the dark overnight at 4 °C following the addition of scintillation cocktail. Radioactivity was then measured using the scintillation counter in dual mode (^{3}H/^{14}C). Blood concentrations of spherulites and ara-C at the various time points were calculated on the assumption that blood represents 7.5% of rat body weight [41,42]. The mean area under the blood concentrations vs. time curve (AUC), the blood clearance (CL) and other pharmacokinetic parameters were determined using a non-compartmental model with PK Solutions 2.0 software (Summit Research Services, Montrose, CO, USA).

2.8. Statistical analysis

Differences in group means (multiple comparisons) were calculated by standard analysis of variance followed by the Kruskal–Wallis test to determine the significance of all paired combinations. The homogeneity of variances across groups was verified by Dunn's test (modified Nemenyi). A P-value ≤ 0.05 was considered significant.

3. Results

3.1. Spherulite preparation characterization

Spherulite formulations with varying compositions were prepared and analyzed by DLS for size determination (Table 1). Fig. 2B shows the freeze-fracture photomicrograph of a non-diluted sheared lamellar phase presenting the characteristic closely packed spherulite arrangement. Vesicles were obtained within a narrow range of surfactant (Tween or Solutol HS15), phospholipid (Lipoid S-75 or P90) and saline concentrations. Homogeneous multilamellar vesicles with average hydrodynamic diameters ranging between 180 and 340 nm were obtained when the proportion of surfactant and phospholipid comprised between 13–31% and 34–45% (w/w), respectively. As exemplified by formulations 5 to 8, size decreased with surfactant (Solutol HS15) concentration. It was not possible to formulate spherulites with diameters of less than 190 nm with the excipients listed in Table 1. Indeed, at very high surfactant concentrations (i.e. formulations 1 and 2), an isotropic effect was observed under polarized light microscopy. At the same concentration,

Solutol HS 15 provided spherulite formulations with a slightly greater size than Tween 80 (compare formulations 4 and 6) and Tween 20 (compare formulations 10 and 11). The vesicles could withstand the addition of Chol up to 10% (w/w) (vs. total lamellar phase components including the saline) or 27 mol% (vs. lipid/surfactant). At 20% (w/w) (48 mol%) no spherulites were formed.

In order to avoid any interference with lamellar phase formation during the preparation process and to allow insertion of DSPE-PEG in the outer leaflet only, the lipid-PEG derivative was added to preformed spherulites after the dilution step. The proportion of PEG in the formulation corresponded to 10 mol% of surface-exposed phospholipids or 0.6 mol% of the total phospholipid content. The amount of PEG added was calculated on the assumption that 14.4±3.5 mol% of phospholipids were present on the external monolayer as determined by the TNBS assay. This value correlates well with that obtained by simple geometrical calculations for 200-nm spherulites (10% of the lipids) [29]. Decoration of the vesicles with PEG was not associated with a noticeable increase in diameter (data not shown), reflecting the difficulty to detect by DLS small size increments (5–7 nm) [37] for particles greater than 200 nm.

3.2. In vitro release of the fluorescent dye and ara-C

The release of HPTS was monitored over time for 3 different spherulite formulations (Fig. 3). The release profile obtained for sample 4 (P90/Tween 80, 76:24 mol%) was characterized by a fast initial release and loss of 70% of the vesicle content within 7 min. The burst effect was significantly attenuated when Tween 80 was substituted for Solutol HS15 (formulation 9), and almost abolished after the incorporation of 27 mol% Chol (formulation 11). For these 2 systems, the release rate was almost constant and corresponded to 3.6 and 0.3%/h, respectively. However, when incubated in 50% (v/v) plasma, the HPTS leakage from the formulation 11 increased significantly and reached approximately 50% after 3 h. Formulation 11, which exhibited the lowest leakage in vitro, was selected for further studies involving the anticancer drug, ara-C. Fig. 4 shows the size exclusion chromatograms of both [^{3}H]-ara-C and [^{14}C]-labelled spherulites. The EE of ara-C was of 46±1% before the PEGylation step. After 15 min incubation in 50% (v/v) fresh rat serum at 37 °C, uncoated, PEG 2000- and PEG 5000-coated spherulites released 57±6, 50±2 and 43±4% of their cargo, respectively ($P \leq 0.05$ for PEG 5000 vs. control). DLS analysis of the spherulite fraction revealed no change in vesicle mean size in the presence of serum (data not shown).

3.3. Pharmacokinetic and biodistribution

The pharmacokinetics and biodistribution of uncoated and PEGylated spherulites containing 0.9% (w/w) ara-C after purification, were assessed following IV administration to Sprague–Dawley rats (Fig. 5A). Uncoated spherulites were rapidly cleared from the systemic circulation. Only 10% of the injected dose remained in the bloodstream 5 min after injection. As expected, the addition of PEG to the formulations significantly improved the circulation times. Indeed, it took 4 h and 12 h to eliminate 90% of the injected dose for PEG 2000- and PEG 5000-coated vesicles, respectively. As shown in Table 2, PEGylation induced a 3.1–6.9 increase in AUC_{0-24h} vs. the control formulation ($p \leq 0.05$). CL values also decreased accordingly. Fig. 5B depicts the deposition of the carriers in various organs 24 h post-injection, a time point at which all formulations were

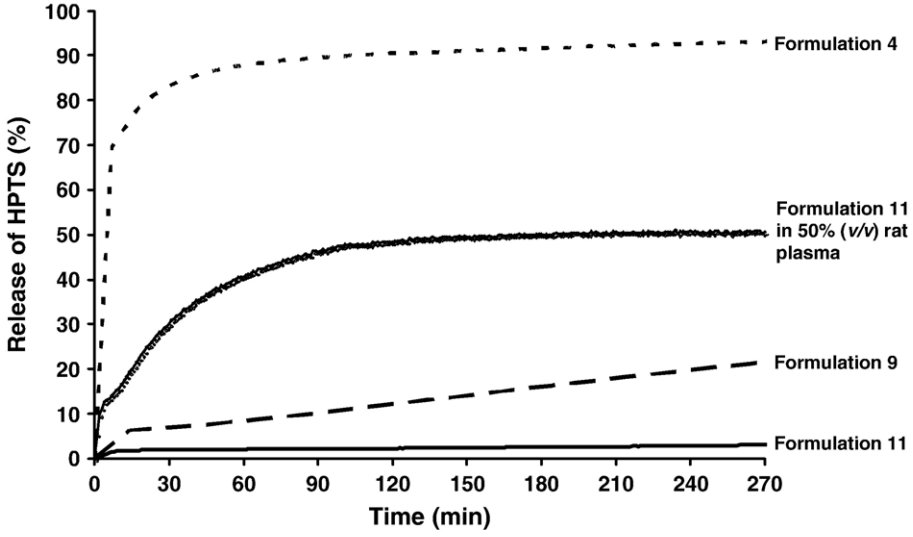

Fig. 3. Release rate of encapsulated HPTS for formulations 4 (P90/Tween 80, 76:24 mol%, dotted line), 11 (P90/Solutol HS15/Chol, 58:15:27 mol%, solid line), and 9 (P90/Solutol HS15, 66:34 mol%, dashed line) in HEPES buffer at 37 °C. The leakage of HPTS for the formulation 11 in 50% (v/v) rat plasma/HEPES buffer at 37 °C is also presented as a function of time (bold dotted line). The extent of content release was calculated from HPTS fluorescence intensity (λ_{ex}=413 nm, λ_{em}=512 nm) relative to measurement after vesicle disruption in 0.9% (v/v) Triton X-100.

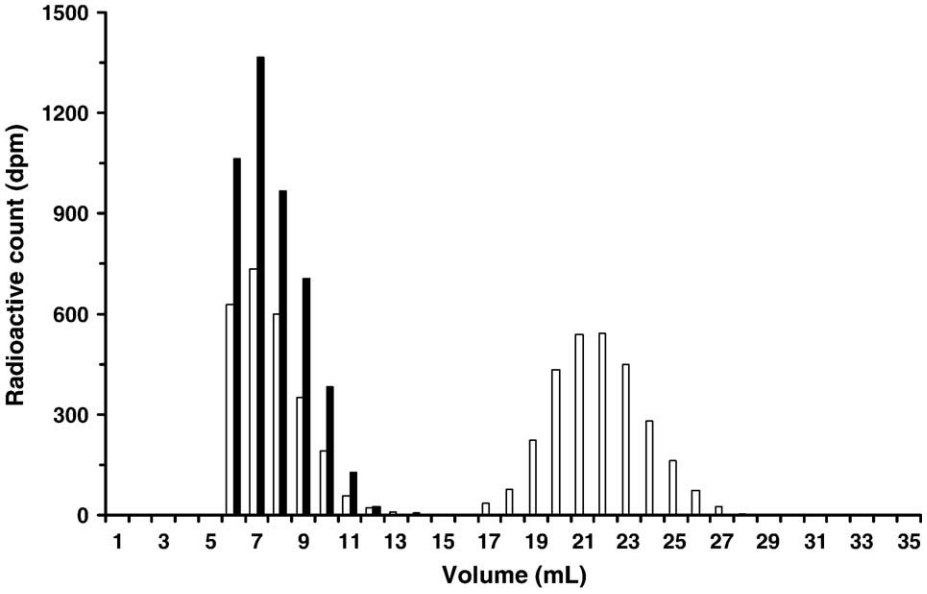

Fig. 4. Elution profile of ara-C (open bars) and spherulites (P90/Solutol HS15/Chol, 58:15:27 mol%, closed bars) after passage over a Sephadex G50 column. The spherulites were labelled with 57 pCi/mg [^{14}C]-cholesteryl oleate and initially loaded with 2% drug (w/w) spiked with 114 pCi/mg [^{3}H]-ara-C. 46 ± 1% of ara-C was entrapped in the vesicles (n = 3).

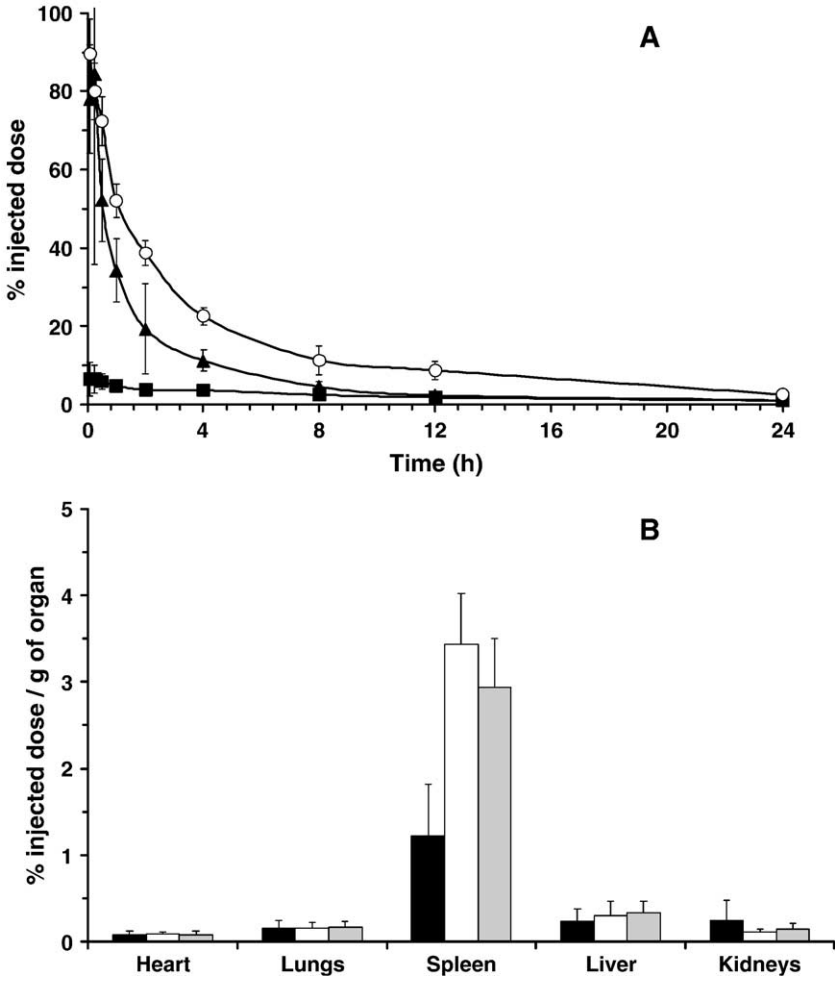

Fig. 5. Blood concentration-time profiles (A) and tissue distribution 24 h post-administration (B) of uncoated (■, black bars), PEG 2000-(▲, white bars) and PEG 5000-coated spherulites (○, grey bars) (P90/Solutol HS15/Chol/DSPE-PEG, 57.4:14.8:27.2:0.6 mol%), after IV administration to rats. Spherulites were labelled with 0.25 μCi/mg [^{14}C]-cholesteryl oleate. Each rat received 0.33 μmol/kg lipids/surfactant. Mean ± S.D. (n = 5).

Table 2
Mean pharmacokinetic parameters of spherulites and ara-C, free or encapsulated in uncoated and PEGylated spherulites (P90/Solutol HS15/Chol/DSPE-PEG, 57.4:14.8:27.2:0.6 mol%), after bolus IV administration to rats

	$AUC_{0\text{-}24\ h}$ spherulites (μg min/mL)	AUC∞ spherulites (μg min/mL)	T½ spherulites (min)	CL spherulites (mL/min)	$AUC_{5\text{-}120}$ min ara-C[a] (μg min/mL)
Free ara-C	–	–	–	–	14.0±1.3
Uncoated spherulites	2 203±217	3 038±449	<5	3.4±0.3	12.8±4.2
Spherulites-DSPE-PEG 2000	6 860±1 120	7 497±1 171	50±14	1.1±0.2	42.4±16.9
Spherulites-DSPE-PEG 5000	15 207±2 490	16 760±3 008	128±30	0.5±0.1	69.2±11.0

Each data point is mean±S.D. ($n=5$ rats/group).
[a] The other pharmacokinetic parameters of ara-C could not be determined due to lack of data points within the first 2 h post-injection.

eliminated from the bloodstream. Irrespective of their composition, the spherulites accumulated mainly in the organs of the MPS (liver and spleen), with the spleen demonstrating the highest vesicle concentration.

In parallel to the spherulite pharmacokinetics, the blood profiles of encapsulated ara-C were also monitored over time (Fig. 5A). The formulations were compared to a solution of ara-C in saline. Both the free drug and ara-C entrapped in control spherulite formulation were rapidly eliminated, with approximately 5% of the injected dose remaining in the bloodstream 5 min after administration. As shown in Fig. 6A and Table 2, the drug encapsulated in the PEGylated vesicles exhibited a 2.6- to 4.8-fold increase in $AUC_{5-120min}$ ($P \leq 0.05$ for PEG 5000 vs. control and free drug). However, 2 h after injection, ara-C was completely cleared from the systemic circulation irrespective of the

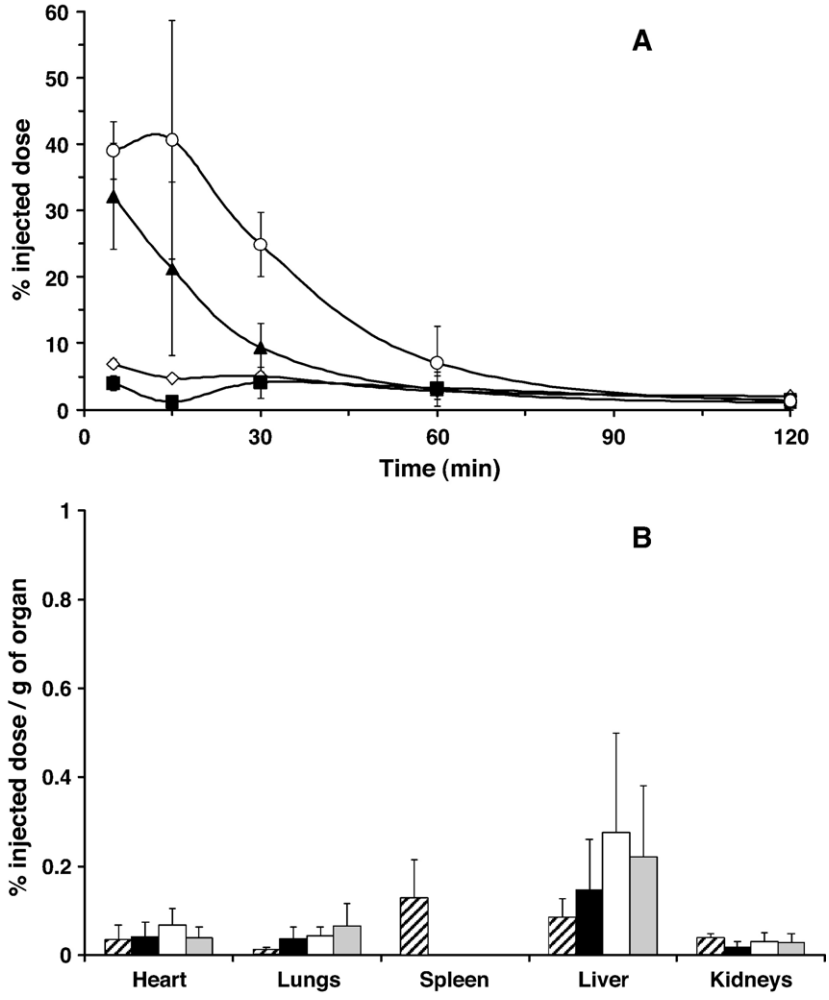

Fig. 6. Blood concentration-time profiles (A) and tissue distribution 24 h post-administration (B) of free ara-C (right dashed bars) and ara-C loaded in uncoated (■, black bars), PEG 2000-(▲, white bars) and PEG 5000-coated spherulites (○, grey bars) (P90/Solutol HS15/Chol/DSPE-PEG, 57.4:14.8:27.2:0.6 mol%) after IV administration to rats. Spherulites were initially loaded with 2% (w/w) ara-C spiked with 100 μCi/mL [^{3}H]-ara-C. Free ara-C was removed by gel filtration over Sephadex G50. Each rat received 2.34 μg/kg ara-C. Mean±S.D. ($n=5$).

formulation. The encapsulated drug mainly accumulated in the liver (Fig. 6B). As opposed to the spherulites, free ara-C was detected in the spleen (0.13% injected dose/g of organ) at the end of the pharmacokinetic study. For all organs, drug accumulation remained low with less than 0.3% of the administered dose/g of organ found 24 h post-injection.

4. Discussion

A simple method based on manual shearing of a lyotropic lamellar phase was used to prepare MLV composed of PC and surfactants. The first objective of this study was to optimize the spherulite size by modifying the formulation composition so as to obtain a mean hydrodynamic diameter of less than 300 nm with monodisperse size distribution. The surfactants and phospholipids were selected based on their suitability for parenteral use. Spherulites were obtained following the application of an external shear force on well-defined lipid/surfactant/saline mixtures. Under moderate shear rate, the phospholipid/surfactant membranes are broken by the flow and wrapped around a spherical core forming the close-packed spherulite arrangement (Figs. 1 and 2B). In this state, the orientation of the lamellar phase is not oriented with the membrane parallel to the flow direction velocity, as seen at very low and high shear rates [24,27]. One characteristic of this intermediate state is that all spherulites formed under moderate shear rate have the same dimension. Moreover, the vesicle size can be correlated to the shear rate and amphiphile volume fraction [24,43].

As shown in Table 1, the minimal size limit that could be reached was around 200 nm for a maximal surfactant concentration of approximately 30% (w/w). At higher surfactant concentrations, the organized lamellar arrangement was lost as revealed under polarized optical microscopy by the absence of Maltese crosses and the apparition of large aggregates upon dilution (data not shown). Although spherulites with diameters of 100 nm (polydisperse size) have been previously reported in the literature [44], such small vesicles were obtained with amphiphiles (e.g. macrogol oleate) that are not approved for parenteral use. The formulations could withstand the addition of 27 mol% Chol without affecting the structure of the sheared lamellar phase.

The in vitro release kinetics of a model hydrophilic dye (i.e. HPTS) was investigated using three different spherulite formulations with mean diameters of ca. 250 nm. It has been previously reported that the long-term stability of spherulites is limited by the relatively high permeability of the surfactant/lipid bilayers [44]. However, it was found that the nature of surfactant greatly influenced leakage from the spherulites. Indeed, replacing Tween 80 by Solutol HS15 resulted in a strong reduction of the release rate (Fig. 2). As both surfactants display the same hydrophilic–lipophilic balance value (HLB ≈ 15) [45] and same alkyl chain length (C_{18}), the change in permeability might be explained by a difference in membrane packing. The two surfactants have different polar head configurations, and the alkyl chain of Solutol HS 15 is saturated whereas that of Tween 80 is unsaturated. Indeed, it was reported that unsaturated alkyl chains produce a looser packing of liposomal membranes than saturated ones, which results in increased permeability for organic molecules [46–48]. Moreover, we showed that the addition of Chol (27 mol%) in the formulations further reduced dye leakage. This was expected since incorporation of Chol in lipidic vesicles has been shown to increase packing densities of phospholipid molecules [49], and thus reduce bilayer permeability to aqueous compounds [48,50]. From a pharmaceutical viewpoint, continuous leakage under storage conditions, even at a slow rate, is problematic as the spherulites would progressively empty their content. However, an interesting feature of the system is that the sheared lamellar phase can be stable for several months before the dilution step [31]. Therefore, in order to prevent excessive drug leakage during storage, the formulation could simply be diluted extemporaneously prior to its administration. In biological fluids, the spherulites were found to be relatively leaky, which is a concern when a long circulation time is sought.

As PEG was reported to protect the bilayer membrane from destabilization by lipoproteins [7,51], its ability to reduce the diffusion of encapsulated molecules was examined both in vitro and in vivo for the spherulites composed of P90/Solutol HS15/Chol (58:15:27 mol%, formulation 11). The anticancer drug ara-C was entrapped in these spherulites with 46% efficiency. This entrapment yield compared advantageously to those reported for reverse-phase evaporation vesicles, MLV and small unilamellar vesicles, with EE of 15–20%, 5–7%, and 1–2%, respectively [19]. Moreover, liposomes with the same composition (P90/Solutol HS15/Chol; 58:15:27 mol%) and size (270 nm) prepared by lipid hydration followed by extrusion yielded an EE of only 16% (data not shown). The relatively high EE obtained with spherulite technology can be explained by the preparation process. The latter allows the formation of a concentrated lamellar phase where the spherulites are in contact with each other and separated by only a thin aqueous layer at the vesicle junction [25] (Fig. 2B). If a hydrophilic molecule is dissolved in the lamellar phase prior to applying the shear, it will be inserted in the water layers of the spherulites. However, the EE for ara-C was lower than expected, as yields greater than 80% were previously reported for macromolecules, such as proteins or DNA [26,27]. Drug loss may occur by fragmentation of the most external layers during the dispersion process [52] and may be more important for smaller vesicles and molecules with low molecular weight.

Incubation of ara-C-loaded spherulites in 50% (v/v) rat serum was accompanied by a substantial loss of drug

(almost 50% within 15 min), which was slightly lower for the PEGylated formulations. For the same incubation time and in the presence of blood proteins, the leakage of ara-C from uncoated spherulites was higher than for HPTS, probably due to the lower MW of the drug. Regarding the coated vehicles, it has been reported that PEG-lipid derivatives, when incorporated in appropriate concentrations, increase the lipid packing order and reducing the leakage of encapsulated hydrophilic substances [5,6,53]. The effect of PEG-5000 in diminishing drug leakage was significant compared to the control (43 vs. 57%). Still, even PEGylated spherulites may readily interact with small amphiphiles (e.g., lysolecithins, peptides, and fatty acids) present in serum [51]. In contrast to larger proteins, these small molecules may exert their destabilizing effect despite the presence of the steric PEG-barrier.

Attachment of PEG to the liposomal surface was repeatedly shown to increase colloidal stability, as well as prolong the lifetime of the liposomes in vivo [54]. An interesting feature of this work was that the amount of PEG added represented only 0.6 mol% of total spherulite components, which is far less than the 5–6 mol% PEG-lipid concentration commonly used in liposome formulations [9,55]. However, as it was incorporated to preformed spherulites, its surface concentration amounted to 10 mol%. At this concentration, PEG provided spherulites and their encapsulated drug with longer circulation times vs. the control formulations. Owing to its greater exclusion volume [56], PEG 5000 was more efficient than PEG 2000, in extending the spherulite half-life (128 vs. 50 min). The PEGylated formulations were cleared more rapidly than stealth 100-nm liposomes composed of partially hydrogenated egg PC/Chol/DSPE-PEG 1900, which exhibit half-lives reaching 15.3 h in rats [11]. This could be partly attributed to the larger size of the spherulites [16] as well as to their composition, which may be more prone to opsonization. Twenty-four hours post-injection, spherulites were found in high proportions in the spleen, reflecting the splenic filtration of large sized (220–300 nm) long-circulating colloids [57]. Likewise, ara-C was associated with a significantly higher blood $AUC_{5-120min}$ when incorporated in PEGylated spherulites. However, 2 h after dosing, the drug was virtually eliminated (Fig. 6A) from the bloodstream, whereas 19–39% of PEG-coated formulations was still circulating (Fig. 5A). These results illustrate the rapid drug loss from the vesicles in biological fluids. This rapid leakage was previously reported for MLV-encapsulated [H^3]-ara-C (PC/Chol/stearylamine), where only 0.86% of the injected dose remained in the bloodstream 3 h after IV administration [58]. However, Allen et al. [59] shown that prolonged circulation time and dose-independent pharmacokinetics have been observed for liposome-entrapped ara-C in mice bearing L1210 leukaemia. They demonstrated that the inclusion of free ara-C in a formulation presenting a slow leakage rate (HSPC/Chol/DSPE-PEG) resulted in a significant improvement in therapeutic effect.

5. Conclusion

This work was the first attempt to apply spherulite technology to the encapsulation of an anticancer drug and presented the first pharmacokinetic study conducted with such PEGylated vesicles. Owing to their simple preparation process and high entrapment efficiency, these vesicles appear as attractive alternative drug delivery system to conventional liposomes. In addition, like liposomes, they can be coated with PEG-lipid derivatives to acquire long circulation times. Future work should now be aimed at minimizing content leakage in biological fluids and further downsizing the vesicles. This could be achieved by changing the phospholipid/surfactant composition (e.g., use of high phase transition lipids) and finely tuning the Chol content.

Acknowledgements

This project was partially funded by the Canada Research Chair Program. We extend our gratitude to Corrine Degert and René Laversanne (Ethypharm) for helpful discussions and their expertise on spherulites and to Marie-Christine Jones and Thomas O'Hare for their critical reading of the manuscript.

References

[1] J.H. Senior, Fate and behaviour of liposomes in vivo: a review of controlling factors, Crit. Rev. Ther. Drug Carr. Syst. 3 (1987) 123–193.
[2] H.M. Patel, Serum opsonins and liposomes: their interaction and opsonophagocytosis, Crit. Rev. Ther. Drug Carr. Syst. 9 (1992) 39–90.
[3] D.D. Lasic, F.J. Martin, A. Gabizon, S.K. Huang, D. Papahadjopoulos, Sterically stabilized liposomes: a hypothesis on the molecular origin of the extended circulation times, Biochim. Biophys. Acta 1070 (1991) 187–192.
[4] S.C. Semple, A. Chonn, P.R. Cullis, Interactions of liposomes and lipid-based carrier systems with blood proteins: relation to clearance behaviour in vivo, Adv. Drug Deliv. Rev. 32 (1998) 3–17.
[5] M. Silvander, M. Johnsson, K. Edwards, Effects of PEG-lipids on permeability of phosphatidylcholine/cholesterol liposomes in buffer and in human serum, Chem. Phys. Lipids 97 (1998) 15–26.
[6] A.N. Nikolova, M.N. Jones, Effect of grafted PEG-2000 on the size and permeability of vesicles, Biochim. Biophys. Acta 1304 (1996) 120–128.
[7] T. Ishida, H. Harashima, H. Kiwada, Liposome clearance, Biosci. Rep. 22 (2002) 197–224.
[8] A. Chonn, P.R. Cullis, Ganglioside GM1 and hydrophilic polymers increase liposome circulation times by inhibiting the association of blood proteins, J. Liposome Res. 2 (1992) 397–410.
[9] G. Blume, G. Cevc, Molecular mechanism of the lipid vesicle longevity in vivo, Biochim. Biophys. Acta 1146 (1993) 157–168.
[10] A. Klibanov, K. Maruyama, A.M. Beckerleg, V. Torchilin, L. Huang, Activity of amphipathic poly(ethylene glycol) 5000 to prolong the circulation time of liposomes depends on the liposome size and is unfavorable for immunoliposomes binding to target, Biochim. Biophys. Acta 1062 (1991) 142–148.
[11] D. Papahadjopoulos, T.M. Allen, A. Gabizon, E. Mayhew, K. Matthay, S.K. Huang, K.-D. Lee, M.C. Woodle, D.D. Lasic, C. Redemann, F.J.

Martin, Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 11460–11464.
[12] A. Gabizon, F. Martin, Polyethylene glycol-coated (pegylated) liposomal doxorubicin, Drugs 54 (Suppl. 4) (1997) 15–21.
[13] R.K. Jain, Transport of molecules across tumor vasculature, Cancer Metastasis Rev. 6 (1987) 559–593.
[14] H.F. Dvorak, J.A. Nagy, J.T. Dvorak, A.M. Dvorak, Identification and characterization of the blood vessels of solid tumors that are leaky to circulating macromolecules, Am. J. Pathol. 133 (1988) 95–109.
[15] O. Ishida, K. Maruyama, K. Sasaki, M. Iwatsuru, Size-dependent extravasation and interstitial localization of polyethyleneglycol liposomes in solid tumor-bearing mice, Int. J. Pharm. 190 (1999) 49–56.
[16] D.C. Litzinger, A.M.J. Buiting, N. van Rooijen, L. Huang, Effect of liposome size on the circulation time and intraorgan distribution of amphipathic poly(ethylene glycol)-containing liposomes, Biochim. Biophys. Acta 1190 (1994) 99–107.
[17] F. Yuan, M. Leunig, S.K. Huang, D.A. Berk, D. Papahadjopoulos, R.K. Jain, Microvascular permeability and interstitial penetration of sterically stabilized (stealth) liposomes in a human tumor xenograft, Cancer Res. 54 (1994) 3352–3356.
[18] T.M. Allen, G.A. Austin, A. Chonn, L. Lin, K.C. Lee, Uptake of liposomes by cultured mouse bone marrow macrophages: influence of liposome composition and size, Biochim. Biophys. Acta 1061 (1991) 56–64.
[19] F. Hong, E. Mayhew, Therapy of central nervous system leukemia in mice by liposome-entrapped 1-beta-D-arabinofuranosylcytosine, Cancer Res. 49 (1989) 5097–5102.
[20] L.D. Mayer, M.B. Bally, M.J. Hope, P.R. Cullis, Techniques for encapsulating bioactive agents into liposomes, Chem. Phys. Lipids 40 (1986) 333–345.
[21] N.L. Boman, D. Masin, L.D. Mayer, P.R. Cullis, M.B. Bally, Liposomal vincristine which exhibits increased drug retention and increased circulation longevity cures mice bearing P388 tumors, Cancer Res. 54 (1994) 2830–2833.
[22] C. Witschi, E. Doelker, Residual solvents in pharmaceutical products: acceptable limits, influences on physicochemical properties, analytical methods and documented values, Eur. J. Pharm. Biopharm. 43 (1994) 215–242.
[23] O. Diat, D. Roux, Preparation of monodisperse multilayer vesicles of controlled size and high encapsulation ratio, J. Phys., II France 3 (1993) 9–14.
[24] O. Diat, D. Roux, F. Nallet, Effect of shear on a lyotropic lamellar phase, J. Phys., II France 3 (1993) 1427–1452.
[25] T. Gulik-Krzywicki, J.C. Dedieu, D. Roux, C. Degert, R. Laversanne, Freeze-fracture electron microscopy of sheared lamellar phase, Langmuir 12 (1996) 4668–4671.
[26] N. Mignet, A. Brun, C. Degert, B. Delord, D. Roux, C. Hélène, R. Laversanne, J.-C. François, The Spherulites: a promising carrier for oligonucleotide delivery, Nucleic Acids Res. 28 (2000) 3134–3142.
[27] A. Bernheim-Grosswasser, S. Ugazio, F. Gauffre, O. Viratelle, P. Mahy, D. Roux, Spherulites: a new vesicular system with promising applications. An example: enzyme microencapsulation, J. Chem. Phys. 112 (2000) 3424–3430.
[28] O. Freund, J. Amédée, D. Roux, R. Laversanne, In vitro and in vivo stability of new multilamellar vesicles, Life Sci. 67 (2000) 411–419.
[29] P. Chenevier, B. Delord, J. Amédée, R. Bareille, F. Ichas, D. Roux, RGD-functionalized Spherulites as targeted vectors captured by adherent cultured cells, Biochim. Biophys. Acta 1593 (2002) 17–27.
[30] T. Pott, D. Roux, DNA intercalation in neutral multilamellar membranes, FEBS Lett. 511 (2002) 150–154.
[31] F. Gauffre, D. Roux, Studying a new type of surfactant aggregate ("spherulites") as chemical microreactors. A first example: copper ion entrapping and particle synthesis, Langmuir 15 (1999) 3738–3747.
[32] O. Freund, P. Mahy, J. Amedee, D. Roux, R. Laversanne, Encapsulation of DNA in new multilamellar vesicles prepared by shearing a lyotropic lamellar phase, J. Microencapsul. 17 (2000) 157–168.
[33] O. Freund, Biodistribution and gastrointestinal drug delivery of new lipidic multilamellar vesicles, Drug Deliv. 8 (2001) 239–244.
[34] M.J. Keating, K.B. McCredie, G.P. Bodey, T.L. Smith, E. Gehan, E.J. Freireich, Improved prospects for long term survival in adults with acute myelogenous leukemia, J. Am. Med. Assoc. 248 (1982) 2481–2486.
[35] J. Borsa, G.R. Whitmore, F.A. Valeriote, D. Collins, W.R. Bruce, Studies on the persistence of methotrexate, cytosine arabinoside, and Leucovorin in the serum of mice, J. Natl. Cancer Inst. 42 (1969) 235–242.
[36] B.C. Baguley, E.M. Falkenhaug, Plasma half-life of cytosine arabinoside (NSC-63878) in patients treated for acute myeloblastic leukaemia, Cancer Chemother. Rep. 55 (1971) 291–298.
[37] P.S. Uster, T.M. Allen, B.E. Daniel, C.J. Mendez, M.S. Newman, G.Z. Zhu, Insertion of poly(ethylene glycol) derivatized phospholipid into pre-formed liposomes results in prolonged in vivo circulation time, FEBS Lett. 386 (1996) 243–246.
[38] Y. Barenholz, D. Gibbes, B.J. Litman, J. Goll, T.E. Thompson, F.D. Carlson, A simple method for the preparation of homogenous phospholipid vesicles, Biochemistry 16 (1977) 2806–2810.
[39] D.L. Daleke, K. Hong, D. Papahadjopoulos, Endocytosis of liposomes by macrophages: binding, acidification and leakage of liposomes monitored by a new fluorescence assay, Biochim. Biophys. Acta 1024 (1990) 352–366.
[40] P. Moreau, L. Lamarche, A.K. Laflamme, A. Calderone, N. Yamaguchi, J.D. Champlain, Chronic hyperinsulinaemia and hypertension: the role of the sympathetic nervous system, J. Hypertens. 13 (1995) 333–340.
[41] W.A. Ritschel, In vivo animal models for bioavailability assessment, STP Pharma 3 (1987) 125–141.
[42] P. Calvo, B. Gouritin, H. Chacun, D. Desmaële, J. D'Angelo, J.P. Noel, D. Georgin, E. Fattal, J.P. Andreux, P. Couvreur, Long circulating PEGylated polycyanoacrylate nanoparticles as new drug carrier for brain delivery, Pharm. Res. 18 (2001) 1157–1166.
[43] E. van der Linden, T. Hogervorst, Relation between the size of lamellar droplets in onion phases and their effective surface tension, Langmuir 12 (1996) 3127–3130.
[44] M. Genty, G. Couarraze, R. Laversanne, C. Degert, J. Maccario, J.-L. Grossiord, Complex dispersions of multilamellar vesicles: a promising new carrier for controlled release and protection of encapsulated molecules, J. Control. Release 90 (2003) 119–133.
[45] A. Wade, P. Weller, Handbook of Pharmaceutical Excipients, American Pharmaceutical Association, Washington, DC, 1994.
[46] D. Rickwood, B.D. Hames, New Liposomes: A Practical Approach, Oxford University Press, Oxford, 1990.
[47] R. Urquhart, R.Y.S. Chan, Q.-T. Li, L. Tilley, F. Grieser, W.H. Sawyer, w-6 and w-3 fatty acids: monolayer packing and effects on bilayer permeability and cholesterol exchange, Biochem. Int. 26 (1992) 831–841.
[48] R.A. Demel, S.C. Kinsky, C.B. Kinsky, L.L.M. van Deesen, Effects of temperature and cholesterol on the glucose permeability of liposomes prepared with neutral and synthetic lecithins, Biochim. Biophys. Acta 150 (1968) 655–665.
[49] R.A. Demel, B. De Kruyff, The function of sterols in membranes, Biochim. Biophys. Acta 457 (1976) 109–132.
[50] D. Papahadjopoulos, M. Cowden, H. Kimelberg, Role of cholesterol in membranes. Effects on phospholipid–protein interactions, membrane permeability and enzymatic activity, Biochim. Biophys. Acta 330 (1973) 8–26.
[51] H. Du, P. Chandaroy, S.W. Hui, Grafted poly-(ethylene glycol) on lipid surfaces inhibits protein adsorption and cell adhesion, Biochim. Biophys. Acta 1326 (1997) 236–248.
[52] M. Genty, G. Couarraze, R. Laversanne, C. Degert, L. Navailles, T. Gulik-Krzywicki, J.-L. Grossiord, Characterization of a complex dispersion of multilamellar vesicles, Colloid Polym. Sci. 282 (2003) 32–40.
[53] K. Hashizaki, H. Taguchi, C. Itoh, H. Sakai, M. Abe, Y. Saito, N. Ogawa, Effects of poly(ethylene glycol) (PEG) concentration on the

permeability of PEG-grafted liposomes, Chem. Pharm. Bull. (Tokyo) 53 (2005) 27–31.
[54] M.C. Woodle, D.D. Lasic, Sterically stabilized liposomes, Biochim. Biophys. Acta 1113 (1992) 171–199.
[55] K. Maruyama, T. Takizawa, T. Yuda, S.J. Kennel, L. Huang, M. Iwatsuru, Targetability of novel immunoliposomes modified with amphipathic poly(ethylene glycol)s conjugated at their distal terminals to monoclonal antibodies, Biochim. Biophys. Acta 1234 (1995) 74–80.
[56] D. Knoll, J. Hermans, Polymer–protein interactions: comparison of experiment and excluded volume theory, J. Biol. Chem. 258 (1983) 5710–5715.
[57] S.M. Moghimi, H. Hedeman, I.S. Muir, L. Illum, S.S. Davis, An investigation of the filtration capacity and the fate of large filtered sterically-stabilized microspheres in rat spleen, Biochim. Biophys. Acta. 1157 (1993) 233–240.
[58] Y.E. Rahman, K.R. Patel, E.A. Cerny, M. Maccoss, The treatment of intravenously implanted Lewis lung carcinoma with two sustained release forms of 1-b-d-arabinofuranosylcytosine, Eur. J. Cancer Clin. Oncol. 20 (1984) 1105–1112.
[59] T.M. Allen, T. Mehra, C. Hansen, Y.C. Chin, Stealth liposomes: an improved sustained release system for 1-beta-D-arabinofuranosylcytosine, Cancer Res. 52 (1992) 2431–2439.